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(Neo)blast from the past: new insights into planarian stem cell lineages

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Collectively, planarian stem cells (neoblasts) are totipotent and are required for tissue homeostasis and regeneration. Recent work has begun to test the long-standing question of whether all neoblasts have the same potential, or whether they actually represent molecularly distinct subpopulations with distinct tissue restriction. Here, we summarize the current state of the field in neoblast lineage organization. It is clear that at least some neoblasts are totipotent, whereas other neoblasts represent functionally distinct molecular subclasses with restricted potential. In addition to neoblast subclasses, tissue-specific progenitors have also been identified, though their ability to proliferate is largely unknown. Together, neoblast lineage development, subclasses, and cell hierarchies are becoming elucidated, showing the complex regulation required for proper tissue homeostasis and regeneration in planarians.

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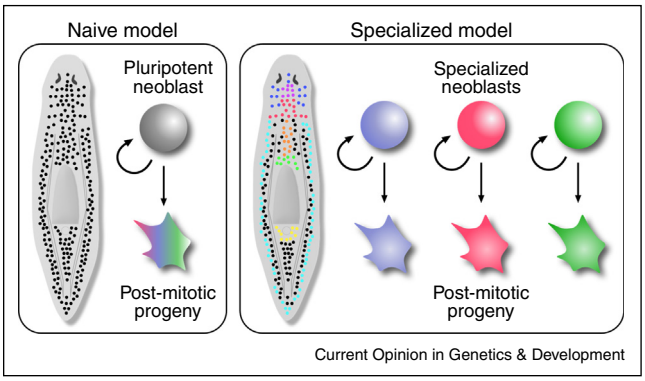
Introduction

Freshwater planarians have long captured the fascination of biologists with their remarkable regenerative abilities [1–3]. While many planarians exist in both sexual and asexual forms, the obligate asexual strain of *Schmidtea mediterranea* has become an important laboratory species due to ease of molecular manipulation and gene knockdown by RNAi [4]. As constitutive adults, *S. mediterranea* tissues undergo constant turnover, they can resize their bodies depending on nutritional intake [5,6], and they can survive massive tissue loss to readily regenerate entire animals from very small amputation fragments (similar to many other planarian species). This biology depends entirely on the activity of neoblasts, a population of mitotically active adult stem cells which can give rise to all cell types in the body (pluripotency). While neoblasts have been classically treated as a single cell type with equal cellular potential, it has been a long-standing question as to how they control the development of their cell lineages in order to replace only the exact cells lost to tissue turnover (homeostasis) or injury, while maintaining the form and function of the animal. This review will examine how recent studies have helped shape our changing ideas of neoblast potential, cellular heterogeneity, and how lineages are organized in planarians.

What are neoblasts?

Neoblasts were historically defined in annelids by the common morphological features of small cell size, a large nucleus to cytoplasm ratio, and the presence of chromatoid bodies [7]. The term was then adapted to planarians to describe similar-looking cells, which constitute 20–30% of the cells in the adult animal [8,9]. In planarians, neoblasts are widely distributed across the anterior–posterior axis and surround the intestinal branches (endoderm) in a mesenchymal-like space known as the parenchyma [9]. Neoblasts are the only mitotic cells in planarians, and are induced after injury to migrate toward the wound and generate post-mitotic descendants to form the regenerative blastema [10,11]. Neoblasts are selectively ablated in 24 hours by gamma irradiation [9,12], which has enabled localization and purification of neoblasts by fluorescence-activated cell sorting (FACS) without prior knowledge of genetic markers [13]. Two irradiation-sensitive populations can be visualized by FACS after Hoechst staining: a high-DNA'X1' gate of cycling neoblasts in S/G2/M phases, and an 'X2' gate containing cells that efflux Hoechst, and is thought to contain a mixture of G1 neoblasts and immediate neoblast division progeny [14•,15•]. From gene expression screening employing irradiation-sensitivity, FACS isolation, or candidate gene approaches, neoblasts can now be molecularly identified by a swath of markers [9,14•,15•,16,17]. Many of these genes are highly conserved in other animals and are typically involved with either cell cycle regulation (*H2B*, *PCNA*, phospho-histone 3) [14•,16,18], or are homologs of germline stem cell markers in other model systems (*piwi-1*, *vasa*, *bruli*, *germinal histone H4*) [9,16,19•,20]. The expression of Argonaute family members in neoblasts is a common trait among all flatworms examined [21–24], and *piwi-1* (*smedwi-1* [9]) is currently used as the gold standard neoblast marker in *S. mediterranea*.

Figure 1



Naïve and specialized models of lineage development. In the naïve neoblast model, neoblasts are a homogeneous population of self-renewing stem cells that are individually pluripotent. Commitment toward different lineages occurs in post-mitotic descendants. In the specialized neoblast model, self-renewing lineage-restricted neoblasts are pluripotent as a collective population. Post-mitotic descendants generated by specialized neoblasts are already committed to specific lineages through parental stem cell identity.

The broad expression of neoblast-specific genes coupled with shared morphological properties has resulted in the traditional treatment of neoblasts as a homogeneous population. However, studies have begun to emerge suggesting lineage decisions can occur within neoblasts, leading to the recent proposal of two models of neoblast lineage development: naïve versus specialized (Figure 1) [25,26]. The naïve model posits that a uniform population of pluripotent stem cells give rise to post-mitotic progeny in which lineage commitment occurs. In the specialized model, neoblasts represent a heterogeneous mixture of lineage-restricted dividing cells which act in concert to remake new tissues. Research in the past five years has now led to the recognition that while planarians do possess individually pluripotent neoblasts, specification toward numerous tissue types begins at the neoblast level.

Establishing pluripotency of individual neoblasts

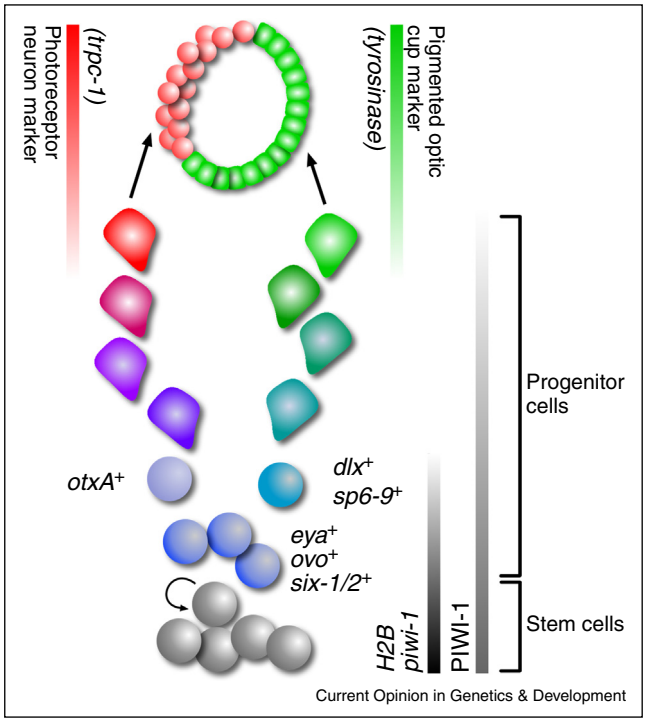
The ability of planarians to remake all tissues from almost any amputation fragment already established that pluripotency was a property of neoblasts at the population level. A seminal study by Wagner and colleagues provided *bona fide* evidence of self-renewal and pluripotency on the level of an individual neoblast [27••]. The authors injected single neoblasts isolated from an asexual strain and found that a subset was capable of repopulating lethally irradiated sexual hosts (clonogenic assay), restoring tissue turnover and regenerative capacity, and ultimately replacing all host cells. Cells capable of this feat were termed clonogenic neoblasts (cNeoblasts), and will likely sit on top of any lineage hierarchy. These results established the existence of totipotent neoblasts at the heart of the naïve model of lineage development, though characteristics of these cNeoblasts still remain a mystery due to lack of prospective isolation, inability to expand them *in vitro*, and inability to genetically label planarian cells. Notably, successful rescue of irradiated hosts occurred in only a fraction of injected neoblasts, suggesting that there may be functional heterogeneity of *piwi-1*$^+$ cells.

Recognition of specialized 'progenitor' cells

Before 2008, the cellular and molecular path between mitotic neoblasts and differentiated functional tissues was unknown. A landmark study performed a microarray time course following lethal irradiation with the hypothesis that stem cell transcripts would be downregulated by 24 hours post-irradiation, and transcripts downregulated between 24 and 72 hours would belong to potential post-mitotic descendants or 'progenitor' cells [14•]. This approach not only found novel stem cell transcripts, but revealed two abundant post-mitotic progenitor cell types, specifically enriched in the X2 FACS gate. Named 'early' and 'late' progeny populations, they sequentially incorporated a BrdU pulse after neoblasts, and were ablated 24–72 hours post-irradiation. Specific markers of early and late progeny showed little overlap with *piwi-1*, and their transient nature indicated they were not fully differentiated; thus, they were the first described progenitors for an unknown lineage, and later confirmed to be in the epidermal lineage [28••].

Upon development of a PIWI-1 antibody, careful experiments showed that the PIWI-1 protein perdures into post-mitotic progeny following down-regulation of *piwi-1* mRNA [16,29]. Using this fact together with staining for transcription factors involved in cell type specification of multiple tissues, it is becoming clear that specification events occur very quickly in lineage development, before PIWI-1 protein disappearance. The term 'progenitors' has been used to describe both *piwi-1*$^+$ and PIWI-1$^+$ cells expressing tissue-specific factors, to reflect their transient gene expression status, apparent commitment to a specific tissue, and proximity to the stem cell in a lineage diagram (Figure 2). In total, progenitor types have been found for the protonephridia [29,30], brain [31–35], pharynx [36], anterior pole/organizer [37,38], and gut [39••]. However, investigations into eye differentiation most clearly illustrate this picture of progenitor maturation [40,41]. These elegant studies showed that spatially restricted *ovo*$^+$ eye progenitors transition from a *piwi-1*$^+$PIWI-1$^+$ neoblast through a *piwi-1*$^-$PIWI-1$^+$ state as they migrate anteriorly toward the eye, and ultimately turn on expression of differentiated genes as they incorporate into the eye proper (Figure 2). While it was clear from these studies that progenitor types exist, it remained unknown whether they derive from a naïve *piwi-1*$^+$ stem cell type, or whether there is high molecular heterogeneity in the stem cell population that reflects the heterogeneity of these progenitor types.

Figure 2



Differentiation of eye lineages. Eye progenitors arise from the pre-pharyngeal region and migrate toward the eye (or eye primordium during regeneration), forming a 'trail' of progressively differentiated cells. Neoblasts (*piwi-1*$^+$/*H2B*$^+$) expressing eye transcription factors are the most posterior eye progenitors detected. It is unknown whether these neoblasts are dedicated self-renewing eye stem cells or continually produced by naïve neoblasts. They begin expressing photoreceptor neuron-associated transcription factors (*otxA*) or pigment cell-associated transcription factors (*dlx*, *sp6-9*), and become *piwi-1*$^-$PIWI-1$^+$ as they migrate anteriorly. Closer to the eye, expression of differentiated eye tissue markers (*trpc-1* or *tyrosinase*) become detectable in the progenitors.
Adapted from [40,41].

Identification of heterogeneity in neoblasts

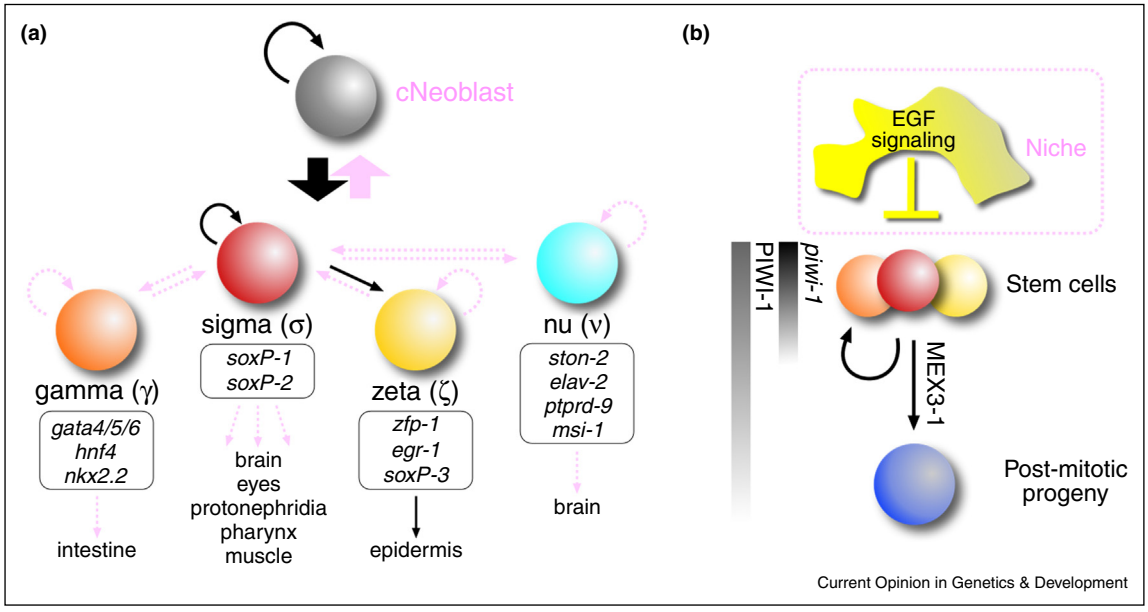
Given the consistent findings of rare *piwi-1*$^+$ or PIWI-1$^+$ progenitors expressing factors involved in differentiation [39••], attention turned to whether functional heterogeneity exists within the neoblast population of cells. The development of single cell transcriptomics paved the way to answering this question. Using a qPCR-based method on single X1 cells with a panel of 96 potential heterogeneously expressed genes, a key study found evidence for three subtypes of neoblasts based on principle component expression clustering [28••]. These neoblast subclasses were termed sigma, zeta, and gamma, and proposed to represent functionally distinct groups (Figure 3a). Though the precise roles of gamma and sigma subclasses have yet to be fully elucidated, specific ablation of zeta neoblasts through knockdown of the zeta subclass marker *zfp-1* has provided sound evidence that this subclass is at least responsible for making the epidermal lineages [28••]. *zfp-1* RNAi abrogated the production of both early and late progeny populations, resulting in impaired homeostatic turnover and regeneration of the epidermis. Interestingly, even without the formation of an overlaying epidermis, blastema formation and regeneration of all other tissue types examined proceeded relatively normally. The specificity of the *zfp-1* RNAi phenotype thus resolved the identity of early and late progeny as epidermal progenitors, and the zeta subclass as a specific class of neoblasts. Although functional data does not exist for the gamma subclass, gamma neoblasts were proposed to give rise to intestinal lineages due to expression of evolutionarily conserved endodermal markers such as *gata4/5/6*. Finally, when bulk neoblast-like cells were transplanted from a *zfp-1(RNAi)* animal into irradiated hosts (i.e. only transplanting non-zeta neoblasts), tissue turnover and regenerative ability could be rescued. Therefore, it is hypothesized that the cNeoblast may reside or be equivalent to the sigma subclass.

A follow-up study by the same group using single-cell RNAseq showed clearer separation between sigma, zeta, and gamma neoblasts; however, it is unknown whether the original panel of subclass markers retain their class-specificity in this new analysis [42•]. Another recent study used single-cell RNAseq to computationally detect neoblasts with neural signatures, called nu-neoblasts, although no functional data yet exists for this subclass [43••]. Without specific ablation or prospective isolation of nu, sigma, or gamma neoblasts, looming questions remain open as to the explicit potentials of each subclass, whether they represent truly different tiers of a cellular hierarchy for neoblast lineages, or whether all three are capable of replacing one another (Figure 3a).

Regulation of neoblast population size: niches

When virtually all neoblasts are removed by sublethal irradiation, the remaining cells symmetrically divide to form expanding colonies. Although neoblast output toward different lineages still occurs during this phase, symmetric expansion continues until the normal complement of neoblasts is restored [19•]. This is highly suggestive of a niche that supports neoblast identity (i.e. is permissive), and once filled, also serves to restrict the size of the neoblast compartment (i.e. restrictive signals). In many stem cell systems, canonical signaling pathways such as WNT, Hedgehog, BMP, and FGF, act in a permissive capacity [44]. However, in planarians, perturbation of these pathways has yielded little effect on the maintenance of a neoblast stem cell state, and permissive signals remain relatively unknown at present [45–51]. Interestingly, the phenotype from disruption of EGFR signaling suggests this pathway may be part of a restrictive niche signal (Figure 3b). *egfr-1* is predominantly expressed in neoblasts and the digestive system, and knockdown of *egfr-1* impairs differentiation of intestinal cell types, resulting in a gradual loss of gut branches.

Figure 3



Neoblast heterogeneity and regulation. **(a)** Four neoblast subclasses have been recently identified [28••,42•,43••]. They are distinguished by expression of specific sets of genes (prominent examples are indicated), and proposed to give rise to different lineages. **(b)** Stem cell population size could be determined by the size of a niche. EGF signaling is a candidate restrictive niche regulatory pathway, and knockdown of *egfr-1* increases the size of the neoblast compartment, although it is unknown whether this signal acts directly on stem cells [52]. Balancing self-renewal and commitment (lineage asymmetry) is another key requirement in maintaining a stable neoblast population. One such component of cell fate determination is *mex3-1*, shown to drive post-mitotic progeny production and differentiation into terminal cell types, while restricting neoblast self-renewal [15•]. Untested cell relationships, functions, or identity are indicated in pink.

Concomitant with the reduction in gut tissue is hyperproliferation and expansion of all neoblast subclasses [52,53•]. A putative ligand, *nrg-1*, was also identified; however, it remains unknown which cell types are the source of the ligand, and whether this EGF signal might act directly on neoblasts. In the future, it will be important to identify the permissive niche signal(s), as well as determine whether it regulates neoblasts in a subclass-specific manner.

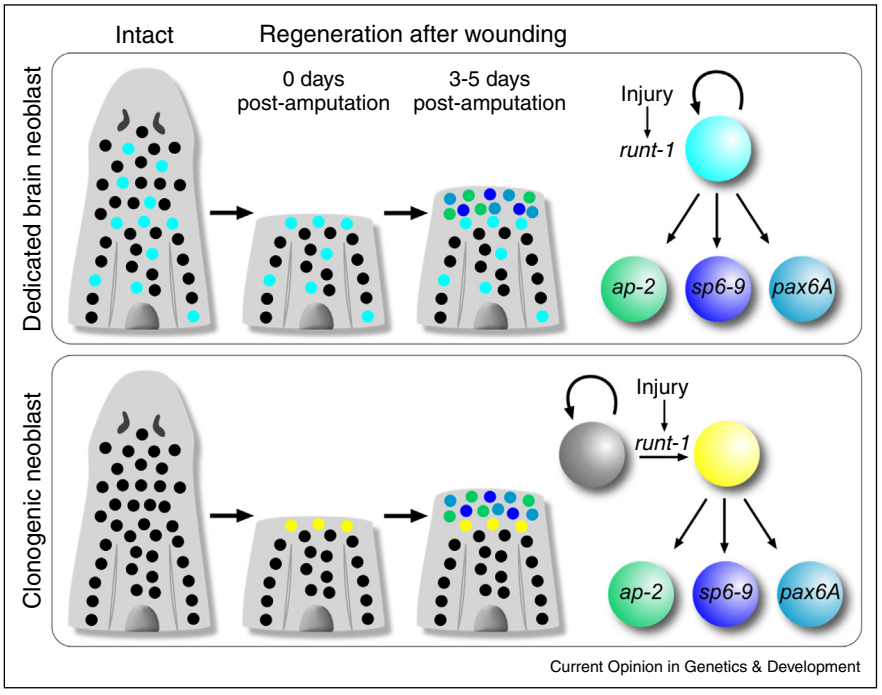
Regulation of neoblast population size: lineage asymmetry

In all adult stem cell systems, in order to maintain a stable cell population size there must be asymmetry in cell fate adoption to balance self-renewal with commitment. It is unknown whether neoblasts undergo asymmetric cell division. No phenotypes have been described for canonical genes involved in asymmetric cell division in other stem cell systems, such as the PAR3-PAR6-aPKC complex, even though these genes are present in planarians. Taking an approach that regulators of progenitor fates may be enriched in the progeny-associated X2 FACS gate, Zhu and colleagues showed that a homolog to the RNA-binding protein MEX3 was a potent cell fate regulator specifically required for differentiation of neoblasts [15•]. Knockdown of *mex3-1*, a gene expressed by neoblasts and progenitors, impaired the generation of all known post-mitotic progenitors and the contribution of newly differentiated cells toward every lineage tested. Concomitant with the decreased post-mitotic output was an expansion of the neoblast pool, which affected all three neoblast subclasses. These data showed that *mex3-1* aids in mediating lineage asymmetry by promoting differentiation and suppressing neoblast self-renewal (Figure 3b). While this study appeared to find a pan-differentiation factor, *mex3-1* was found to be expressed in all *piwi-1*$^+$ cells as well as progenitors. The factors regulating the asymmetric activity of MEX3-1 are still unknown, as are its RNA targets.

Homeostasis versus regeneration: rewriting lineage development?

Wholesale regeneration in planarians poses significant challenges to the above models of lineage development. For example, a very small tail fragment will remake all anterior structures such as eyes, pharynx, organizing poles, and perhaps most dramatically, regenerate a functional brain. How do tail neoblasts, which are not likely to normally make brain cells without injury, regenerate an entire brain de novo? There are two possible ways neoblasts can accomplish this (Figure 4). There may be dedicated brain neoblasts of the nu-subclass scattered across the entire body of the animal, so that any given amputation fragment will inherit at least one to regenerate

Figure 4



Models of brain regeneration after injury. Regeneration of an entirely new brain can occur through the activity of dedicated self-renewing brain neoblasts present throughout the body (teal cells in upper panel), or through the reprogramming of clonogenic neoblasts that, before wounding, would not have otherwise differentiated into brain tissue (yellow cells in lower panel). In either case, it has been shown that injury-induced neoblast expression of *runt-1* is necessary for expression of brain (*ap-2*, *pax6A*) and eye (*sp6-9*) progenitor marker expression [31].

a brain. Alternatively, neoblast identity might be rewritten during regeneration, such that pluripotent neoblasts, inherited in the tail, are provided new instructions to transit through a nu-subclass fate in order to make a new brain. Currently, no dedicated brain neoblasts have been functionally identified that can account for regenerating an entire brain, and several examples support the latter model that neoblast fates can be rewritten. Early in the first 12 hours of head regeneration, anterior signaling pathways are already making the 'head' choice [37,38]. In addition, the *runt-1* transcription factor is expressed in neoblasts only during regeneration, and is required for the de novo expression of neural and eye transcription factors in neoblasts at the anterior wound margin [31,54]. Together, we believe this supports a model that positional information and tissue-specific fates can be rewritten following injury. In future studies, it will be interesting to determine whether specific neoblast subclasses are responsible for regenerating specific tissues, as it appears is the case for homeostasis, or whether cNeoblasts are the key cells to respond and largely remake a given tissue after injury.

Concluding remarks

The last five years have greatly expanded our knowledge of the complexities of neoblast regulation, potency, and commitment to differentiation. However, many more questions remain unanswered. For example, does every tissue type have its own class of dedicated neoblast? Perhaps only select lineages have specialized progenitors, while all others directly differentiate from a naïve neoblast. Going forward, a number of fundamental points will need to be addressed, most likely requiring technological advancements to provide definitive answers, such as genetic labeling or prospective subclass isolation by cell surface antibodies. With additional tools, it will be possible to answer: what is the molecular profile of a cNeoblast? Are all sigma class cells actually cNeoblasts? How interconvertible are these progenitor populations and neoblast subclasses? What cell type(s) provide the permissive niche that maintains the planarian stem cell population size? What are the signaling pathways directing initial specialization of cNeoblasts toward different lineages, and are those completely rewritten during regeneration? Elucidating the mechanisms guiding stem cell lineage development in planarians will aid in the understanding of how adult human stem cells perform similar tasks, and this knowledge will be critical in eventually achieving regenerative therapies in humans.

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